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Phenotypic Variation and Bistable Switching in Bacteria

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12

Phenotypic Variation and Bistable Switching in Bacteria

Wiep Klaas Smits(✉), Jan-Willem Veening, and Oscar P. Kuipers

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Abstract Microbial research generally focuses on clonal populations. However, bacterial cells with identical genotypes frequently display different phenotypes under identical conditions. This microbial cell individuality is receiving increasing attention in the literature because of its impact on cellular differentiation, survival under selective conditions, and the interaction of pathogens with their hosts. It is becoming clear that stochasticity in gene expression in conjunction with the architecture of the gene network that underlies the cellular processes can generate phenotypic variation. An important regulatory mechanism is the so-called positive feedback, in which a system reinforces its own response, for instance by stimulating the production of an activator. Bistability is an interesting and relevant phenomenon, in which two distinct subpopulations of cells showing discrete levels of gene expression coexist in a single culture. In this chapter, we address techniques and approaches used to establish phenotypic variation, and relate three well-characterized examples of bistability to the molecular mechanisms that govern these processes, with a focus on positive feedback.

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12.1 Phenotypic Variation

The potential of bacteria to thrive in a variety of ecological niches strongly depends on their genetic content. However, within these niches, environmental conditions such as pH, salinity, temperature, and the availability of nutrients can strongly fluctuate. In addition, bacteria may be faced with interspecies or intraspecies competition in the form of, for instance, antimicrobial peptides.

To survive these kinds of stresses, bacteria adapt their genetic programs, resulting in change of phenotypes to meet the fluctuating environmental conditions. Environmental signals are commonly interpreted by cells via a system of sensor and regulator proteins that result in a change in the transcription profiles (transcriptome) and/or protein expression patterns (proteome) of the cells (other sensing systems also exist in bacterial cells; see Chaps. 7, 8, and 9 for details on some of these systems). These adaptive changes generally last for the duration of the stress, but in some cases may persist for many generations.

Microbial research has traditionally considered bacterial cultures as homogeneous, because they are a clonal population derived from a common ancestor. Before single-cell analyses of bacterial populations, many heterogeneous processes may have gone undetected or may have been discarded as an artifact of the methods used. Single-cell analyses (see Sect. 12.2) show that cells in a culture demonstrate a remarkable level of variation, which roughly can be divided into two categories.

First of all, bacterial populations exhibit a unimodal distribution in gene expression for most genes, because of random fluctuations in transcription and translation (in other words, there is a normal distribution of expression levels around the mean; Fig. 12.1a, curve 2). These fluctuations are referred to as noise (Ozbudak et al. 2002). This general statistical argument already implies that there can be significant differences in levels of gene expression between individual cells.

In addition, several cases have been identified in which only a subpopulation of cells of a culture demonstrates an adaptive phenotype (Smits et al. 2006). This heterogeneity could result in an increased spectrum of niches that the bacteria can use, and, therefore, has the potential to increase the overall fitness of the species. When two clear subpopulations can be identified, each with a normal distribution in gene expression levels, the population is referred to as bistable (Fig. 12.1a and b, curve 3). Bistability has also been described on the phenotypic level as a situation in which two clearly distinguishable cell types are present within the clonal population (Dubnau and Losick 2006).

Consider a situation in which a certain gene is twofold upregulated under a typical stress condition, as measured by a culture-wide reporter such as beta-galactosidase activity. It is possible that the gene demonstrates twofold upregulation in all cells, but if the gene were expressed in a subpopulation of only 10% of the culture, the fold increase in expression in these specific cells would be closer to 11. Clearly, these findings demonstrate that bulk measurements have to be interpreted with care.

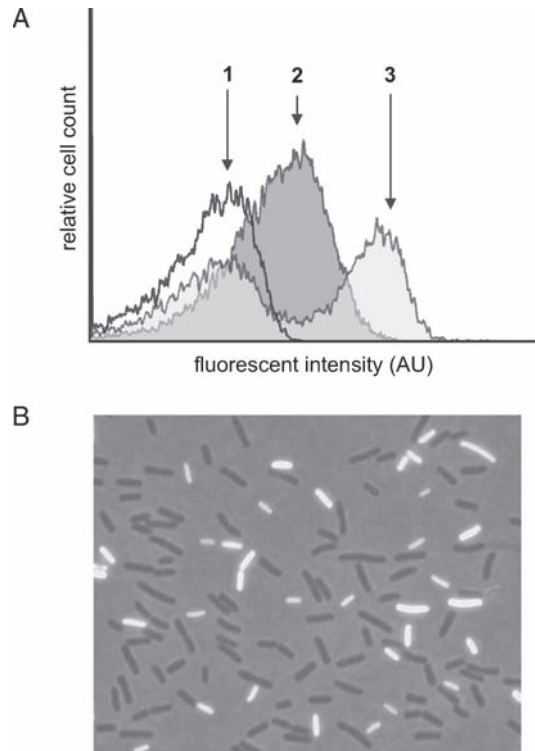


Fig. 12.1 Phenotypic variation. **a** Flow cytometric analysis of bacterial populations carrying a GFP-reporter fusion. Nonfluorescent cells give rise to curve 1. Phenotypic variation is apparent for the populations represented by curves 2 and 3. In the case of curve 2, the underlying distribution in fluorescence is monomodal. Note that some cells may seem nonfluorescent because they overlap with curve 1, depending on the detection limit of the system. Curve 3 represents a bistable expression pattern. Note that the one of the stable states corresponds to nonfluorescent cells. AU, arbitrary units with permission from the publisher. **b** Fluorescence microscopic image of a *B. subtilis* strain harboring a competence-specific GFP reporter. Note two subpopulations of cells, white cells express the reporter, whereas the black cells do not. This figure was adapted from Smits et al. (2006).

12.2 Reporters and Techniques for Single-Cell Analyses

Although culture-wide reporter assays can be informative, the example above demonstrates the necessity to determine whether a response measured in bulk experiments is representative for the events in single cells. To get statistically significant data, large numbers of cells need to be quantified. To this end, several techniques have been developed.

The simplest way to examine single cells is by fixing cells on a (coated) glass slide, followed by visualization using light microscopy. Phase-contrast microscopy suffices in some cases to reveal phenotypic variability. Sporulating cells of *Bacillus subtilis* can readily be distinguished from nonsporulating cells within an isogenic

culture by normal phase-contrast microscopy because sporulating cells contain a readily visible endospore during the later stages of this process, whereas nonsporulating cells do not. However, many subpopulations are not characterized by phenotypes that are distinguishable by phase-contrast microscopy.

As a result, most single-cell reporters rely on fluorescence or luminescence (see Davey and Kell 1996; Kasten 1993). Fluorescence is a physicochemical property of certain molecules and compounds, and can be used to visualize individual biochemical, genetic, or physiological properties (Barer and Harwood 1999; Joux and Lebaron 2000). With the use of specific filters coupled to a light source, such as a mercury or xenon lamp, excitation of fluorophores and detection of fluorescent signals at certain wavelengths can be performed. Microscopic images may be captured using CCD cameras. One of the most powerful ways to examine a large number of cells on their single-cell properties is by flow cytometry. In a flow cytometer, cells pass an intense light source (a laser) one by one, and the data on fluorescence and scatter (which depends on, for instance, particle size) is collected. Depending on the flow cytometer and the settings used, signals from more than 1,000 individual cells per second can easily be determined, making it an extremely valuable technique for time series analyses. Because of its sensitivity and resolution, this is the preferred method to establish whether a heterogeneous culture demonstrates a monomodal or bimodal (bistable) distribution in gene expression levels (see Sect. 12.5.2 and Chung et al. 1995; Smits et al. 2005; Veening et al. 2005).

A battery of fluorescent dyes is currently available to visualize biochemical or physiological properties of cells that may differ from cell to cell (see Brehm-Stecher and Johnson 2004). These dyes include substances that nonspecifically stain DNA (e.g., DAPI, ethidium bromide, and Hoechst), membranes/phospholipids (e.g., FM4-64 and Nile-red) or the cell wall (e.g., Gram staining or fluorescently labeled vancomycin) (Daniel and Errington 2003). In addition, a number of probes can be used to measure individual intracellular pH or redox state of a cell (Nebe-von-Caron et al. 2000; Vives-Rego et al. 2000; Haugland 2002). Using Nile-Red and SYTO-13 stains, phenotypic variability with respect to polyhydroxyalkanoates has been reported for *Pseudomonas aeruginosa* (Vidal-Mas et al. 2001).

Fluorogenic substrates for the classic beta-galactosidase reporter gene, for instance, have successfully been used to demonstrate heterogeneity in the expression of a developmental regulator in *Myxococcus xanthus* (Russo-Marie et al. 1993), as well as to show the characteristics of sporulation bistability in *B. subtilis* (Chung et al. 1995; Chung and Stephanopoulos 1995). In addition, they have been used to study the dynamics of gene expression in a single cell using a microfluidic device (Cai et al. 2006).

When conjugated to a fluorescent dye, macromolecules such as antibodies or nucleic acids can be used to visualize protein localization (immunofluorescence) or the presence of specific RNA or DNA types (fluorescence in situ hybridization [FISH]) (for reviews, see Amann et al. 1995; Davey and Kell 1996; Moter and Gobel 2000; Amann and Ludwig 2000; Brehm-Stecher and Johnson 2004). In short, labeled probes are introduced to the cell, where they bind to target sequences. The high sensitivity of the method makes it possible to detect single molecules, and this method

has been used to demonstrate transcriptional bursting in *E. coli* (Golding and Cox 2004; Golding et al. 2005). Alternatively, in situ reverse transcriptase (RT) polymerase chain reaction (PCR) can be used to amplify the signals in a quantitative manner. Using this technique, nongenetic heterogeneity was demonstrated for *Salmonella* (Tolker-Nielsen et al. 1998) and *Methanosarcina* (Lange et al. 2000), for instance. It has to be noted that RT-PCR can also be used to demonstrate genetic differences between single cells, as, for instance, in the case of phase-variable phenotypes.

All of the fluorescence-based techniques described above rely on the introduction of a probe into the cells, which might pose problems. A major breakthrough in molecular biology has been the use of fluorescent proteins in vivo. The green fluorescent protein (GFP), which was originally isolated from the jellyfish *Aequorea victoria*, offers a noninvasive reporter for gene activity and is now commonly used by microbiologists (Tsien 1998; Southward and Surette 2002).

Modified variants of the protein with distinct excitation and emission spectra have been developed for use in bacterial research (Shaner et al. 2005; Shaner et al. 2004), allowing the visualization of more than one protein or promoter activity simultaneously.

Using GFP and its spectral derivatives to study transcriptional regulation, phenotypic variation was demonstrated, for instance, for the expression of the structural gene *e* for colicin K in *E. coli*, and genomic islands in *Salmonella* (Hautefort et al. 2003) and *Pseudomonas* (Sentchilo et al. 2003a; Sentchilo et al. 2003b). In addition, it was shown that an exponentially growing culture of *B. subtilis* consists of two subpopulations that differ in the expression of swarming/motility genes (Kearns and Losick 2005).

It has to be noted that when the *gfp* gene is translationally coupled to a gene of interest, it can be used to ascertain the subcellular localization of the encoded protein. Such studies have led to many groundbreaking discoveries in bacteria, including the presence of a so-called “replisome” (Lemon and Grossman 1998) and the presence of a dynamic bacterial cytoskeleton (Carballido-Lopez 2006). Additionally, they may reveal another layer of complexity with respect to phenotypic heterogeneity, because the subcellular localization of certain proteins may differ between subpopulations. It was shown, for instance, that RecA localizes to the polar competence machinery of *B. subtilis* cells, whereas it is associated in nucleoids in noncompetent cells (Kidane and Graumann 2005). Because competence occurs in a subpopulation of cells (see Sect. 12.5.2), the localization pattern of RecA is similarly heterogeneous.

Bioluminescence reporters based on the *lux* system, although in principle suitable for single-cell analyses, are less frequently used because the resolution is inferior to fluorescence. Their primary applications are in biosensors, host–pathogen interaction, and the study of circadian rhythms (Greer III and Szalay 2002).

Some cellular characteristics are more easily assessed by alternative techniques. The study of surface properties of single cells is greatly facilitated by microcapillary electrophoresis (reviewed in Brehm-Stecher and Johnson 2004). Surface properties may depend on cell age, cell cycle status, or cell type, and are, therefore, of interest to a broad field of researchers (Glynn Jr et al. 1998). Using capillary electrophoresis, it was shown that cultures of *Enterococcus faecalis* demonstrate heterogeneity in

surface charge, which affects adhesion and biofilm formation (van Merode et al. 2006a; van Merode et al. 2006b).

Finally, cell density centrifugation deserves to be mentioned. Although technically not a single-cell technique, subpopulations with discernible buoyant density can be separated and analyzed after recovery. This type of analysis has revealed subpopulations during growth of *E. coli* (Makinoshima et al. 2002) and *Vibrio parahaemolyticus* (Nishino et al. 2003), and genetic competence of *B. subtilis* (Hadden and Nester 1968; Cahn and Fox 1968; Dean and Douthit 1974; Haijema et al. 2001).

12.3 Sources of Phenotypic Variation

The origins of different cell types in a culture can be as diverse as the phenotypes the cells display, and can be both genetic and nongenetic in nature.

In the stationary-growth phase, bacteria can display *adaptive mutagenesis*. Several genetic determinants for this process have been identified, such as DNA duplications and the expression of genes involved in DNA metabolism and genome integrity. When the mutations affect certain bacterial properties, they may lead to a growth advantage of certain cells over others (Finkel and Kolter 1999). As a result, one phenotype may overgrow the other, potentially leading to the dynamic coexistence of multiple phenotypes.

Another well-characterized mechanism to generate phenotypic variation is phase variation (for review, see Henderson et al. 1999). Phase-variation phenotypes show a binary pattern, in which genes are expressed (ON) or not expressed (OFF). Transitions between the two states are random, and occur at a relatively high frequency ($>10^{-5}$ per generation). Phase variation resembles antigenic variation of pathogenic bacteria in many aspects, although these are distinct processes. The best-characterized examples of phase variation involve cellular appendages such as fimbriae and flagellae. Phase variation frequently depends on genetic changes, such as genomic inversions or strand-slippage mechanisms. For instance, the phase-variable expression of both type I fimbriae in *E. coli* (Abraham et al. 1985) and flagellae in *Salmonella* (Zieg et al. 1977) involve the inversion of a DNA element (Fig. 12.2a), whereas the expression of certain outer membrane proteins in *Neisseria* (reviewed in Meyer et al. 1990) and virulence factors in *Bordetella* (Stibitz et al. 1989) is determined by frameshift mutations as the result of strand slippage (Fig. 12.2b).

In addition to genetic changes, the origin of phenotypic variation may reside in modifications of the DNA (Fig. 12.2c). These changes are epigenetic in nature, because they do not involve a change in DNA sequence, yet the phenotypes associated with the modification can be inherited by daughter cells after division. For instance, the phase-variable expression of the *pap* operon in *E. coli* involves methylation of two GATC sequences by the Dam methylase (van der Woude et al. 1996). In this case, the methylation pattern of the DNA affects the position of binding for a transcriptional regulator, resulting in repression or activation of transcription.

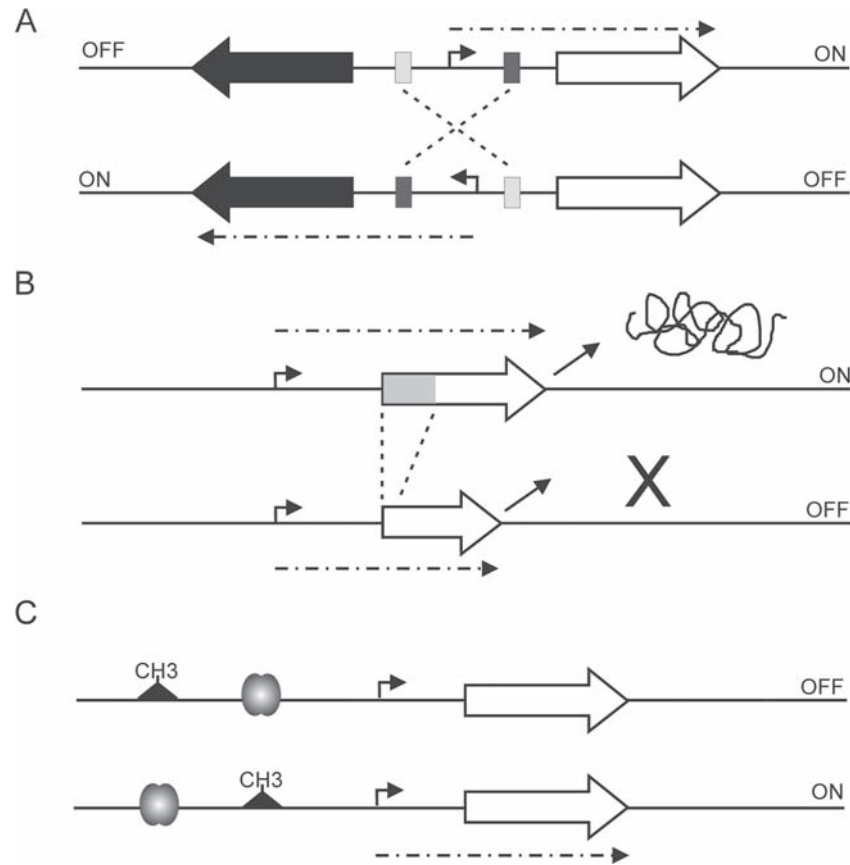


Fig. 12.2 Phase variation. **a** Phase variation as a result of genomic inversion. An element containing a promoter is located in between two divergently oriented genes, driving the expression of one of the genes. After inversion of the element, the other gene is expressed. **b** Phase variation caused by strand slippage. The deletion of a small fragment of the DNA places results in a frame-shift mutation and premature termination of protein synthesis. **c** Phase variation caused by methylation. Methylation of certain sequences of the DNA can prevent the binding of transcriptional regulators, and cause them to bind at different locations. As a result, genes are ON or OFF

However, phenotypic variations need not be accompanied by changes in DNA sequence or modifications of the DNA. Asynchronously growing cells can display different phenotypes depending on the stage of the cell cycle or the growth. Cell cycle-related variation plays an important role in the coordinate expression of genes required for replication and cell division (Holtzendorff et al. 2006; Avery 2006), and has been shown to be responsible for the heterogeneous expression of certain proteins (Sumner and Avery 2002; Rosenfeld et al. 2005). Biological cycles also occur on a longer time scale, as in circadian rhythms (Williams 2007). Because many cellular processes are temporally regulated, cells in different growth phases would automatically result in phenotypic variation at the population level. These

forms of phenotypic variation can be distinguished by the synchronization of starter cultures, as well as by correlation with known markers for the cyclic processes.

Cell age can add another level of complexity (Avery 2006). Recently, elegant data were collected suggesting that even symmetrically dividing organisms such as *E. coli* suffer from aging, because cells with older cell poles show lower growth rates compared with cells that have inherited more new poles (Stewart et al. 2005). Although the mechanistic details regarding the role of aging in phenotypic variation are unknown, it implies that cell aging also needs to be taken into account for bacterial research.

It was already pointed out in Sect. 12.1 that gene expression in a homogeneous culture displays a normal distribution in gene expression levels, because of stochastic events (noise). Because, in the absence of a stimulus, inducible transcription factors are, in general, low abundant proteins, it is noteworthy that noise is more significant when fewer molecules are involved (finite number effect). It was shown that noise is most pronounced in the case of low transcription coupled with high translation (Ozbudak et al. 2002), although both transcription and translation levels can have a significant influence. Total noise levels can be broken down into intrinsic noise and extrinsic noise (Swain et al. 2002). Intrinsic noise is inherent to the biochemical process of gene expression, whereas extrinsic noise can be attributed to external factors acting on a single cell (Elowitz et al. 2002). The two types of noise can be discriminated using a system of distinguishable fluorescent reporters that are driven from the same promoter but are located at different positions on the chromosome; intrinsic noise will be reflected by a broad range of ratios in the fluorescence levels of the two reporters in single cells, whereas, for extrinsic noise, the ratios will be similar but the absolute levels of fluorescence may differ per cell (Fig. 12.3). Fluctuations caused by the different types of noise demonstrate different characteristics: intrinsic noise causes rapid fluctuations, whereas extrinsic noise causes fluctuations on a larger time scale (Rosenfeld et al. 2005). Finally, although both types of noise can lead to similar population distributions, it has been reported that, overall, extrinsic noise contributes more to phenotypic variation than intrinsic noise (Elowitz et al. 2002). It is of importance to realize that noise can be propagated in a gene network and that this can significantly affect the final output of a regulatory cascade (Pedraza and van Oudenaarden 2005).

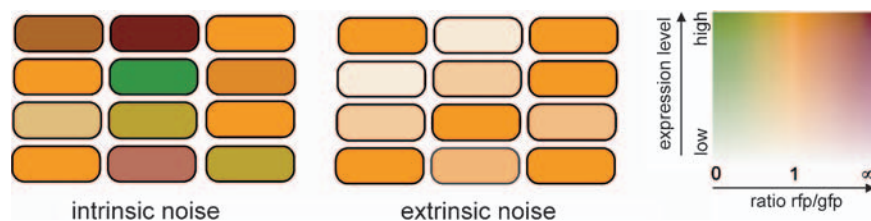


Fig. 12.3 Noise in gene expression. Intrinsic noise results in a wide range of ratios (from red to green) between the two distinguishable reporter constructs that are driven from the same promoter. Extrinsic noise results in fluctuations in the levels of transcription (from dark to light), whereas the ratio between the two reporters (in this case equal to 1) is the same for all cells. Rfp, red fluorescent protein

In general, high levels of noise are regarded as detrimental for the fitness of cells. In accordance with this, essential genes were reported to exhibit lower levels of noise than non-essential genes (Fraser et al. 2004). One of the most ubiquitous and efficient ways to control the level of noise in gene expression is the introduction of a negative feedback loop (Becskei and Serrano 2000). By limiting the production as the levels of a certain protein increase, negative autoregulation limits the range over which fluctuations in the level of this protein can occur. In addition to this, the intertwinement of regulatory networks can have the ability to filter noise (Hooshangi et al. 2005).

In specific cases, however, noise is exploited to generate phenotypic variability (Rao et al. 2002, Hasty 2000). For example, it was shown that noise in the regulatory cascade governing the chemotactic response of *E. coli* is responsible for behavioral variability of individual cells, as measured by the rotational direction of flagella (Korobkova et al. 2004). Importantly, noise can be amplified through the introduction of a positive feedback loop, resulting in bistability (or more universal: feedback-based multistability [FBM]; see Sect. 12.4 and Smits et al. 2006).

12.4 Bistability in Gene Expression

Here, we refer to bistability as the coexistence of two distinct subpopulations of cells in a culture that does not depend on modifications of the DNA. Each of these subpopulations represents a discrete level of gene expression, referred to as a state. If a gene regulatory network can lead to more than a single stable state, it is said to exhibit multistationarity. Thus, multistationarity at the cellular level can result in a bistable (or multistable) bacterial population (Smits et al. 2006). Switching between these states occurs stochastically, and is usually reversible.

As early as the 1960s, it was postulated that feedback regulation might be responsible for the stable states observed for all-or-none enzyme induction (Novick and Weiner 1957; Monod and Jacob 1961). Pioneering work of Thomas has highlighted the importance of feedback regulation for multistationarity (Thomas 1978, 1998). Subsequently, the prerequisites for a gene network to generate a multistable output were determined by mathematical modeling and the analysis of artificial gene networks (Angeli et al. 2004; Ferrell Jr 2002; Friedman et al. 2006; Gardner et al. 2000; Kobayashi et al. 2004; Hasty et al. 2000; Hasty et al. 2002; Isaacs et al. 2003).

It was found that the system needs to exhibit nonlinear kinetics, implying that the response (e.g., the expression of a reporter gene) is not a linear function of the concentration of a regulator (Ferrell Jr 2002). Frequently, this is apparent from the observation of a threshold concentration of the regulator to elicit a response (Chung et al. 1994). For transcriptional regulators, nonlinearity can for instance be observed as a result of the requirement for multimerization, cooperativity in DNA binding, or phosphorylation of certain amino acid residues.

Without the introduction of complicated mathematical models, the effects of single positive feedback can easily be envisaged (Fig. 12.4). In the absence of

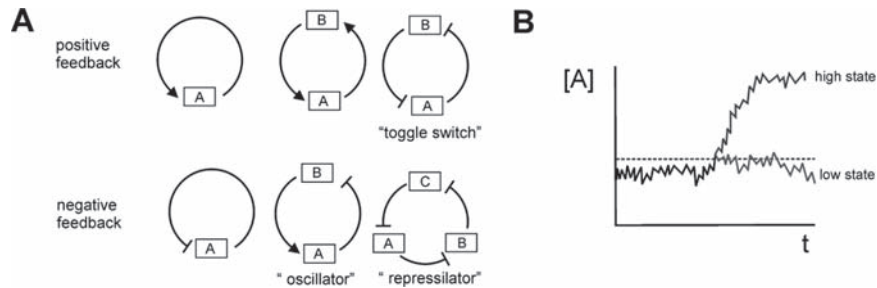


Fig. 12.4 Gene networks. **a** Effective positive feedback can be established by, for instance, a one-component or a two-component positive feedback loop, or a two-component negative feedback loop (toggle switch). Arrowheads indicate a positive effect, perpendicular lines a negative effect. Negative feedback is displayed by networks harboring a single negative feedback loop. A combination of positive and negative interactions in a two-component loop (oscillator) as well as a three-component negative feedback loop (repressilator) can cause a system to oscillate between states. **b** The effect of positive feedback on noise-induced fluctuations in protein level in time. In the presence of a positive feedback loop, expression levels will increase to the maximum level (high state), whereas, in the absence of positive feedback, fluctuations remain within the low state (**b**, adapted from Smits et al. 2006) with permission of the publisher.

autostimulation, an increase in the level of a regulator will result in a concomitantly increased response (graded response). Autostimulation requires a threshold concentration of a regulator. As a result of noise, certain cells will reach the threshold, initiating a positive feedback loop and causing cells to switch between a low- and high-expressing state (Fig. 12.4b) (Ferrell Jr 2002; Isaacs et al. 2003). Indeed, it was experimentally verified that the introduction of an autostimulatory loop can convert a graded response into a bistable one (Becskei et al. 2001). Increasing the level of the autostimulatory regulator (either by increasing its expression or its activity) will cause more cells to switch from the low to the high state, without affecting the position of the peaks in the bistable distribution pattern (Becskei et al. 2001; Smits et al. 2005; Veening et al. 2005).

Similar behavior is observed with a system of two activators that stimulate the expression of each other. In this double-positive feedback loop, regulator A will stimulate the expression of regulator B once a certain threshold has been reached. In the resulting stable state, the levels of A and B are both high.

A system of two regulators that repress each other, known as a toggle switch, also exhibits two stable states (Ferrell Jr 2002; Tian and Burrage 2004; Lipshtat et al. 2006). As the concentration of regulator A reaches the threshold for repression of gene B, it will indirectly activate its own transcription. The two stable states for a toggle switch, therefore, correspond to reciprocal states of A and B; the presence of high levels of regulator A excludes high levels of B, and vice versa. Experimentally, the functionality of a toggle switch was shown in *E. coli* (Gardner et al. 2000; Kobayashi et al. 2004). The strength of the regulatory interactions is critical, because a toggle switch-like module will not result in multistationarity if one of the components acts much stronger than the other.

Importantly, only gene networks that demonstrate net positive feedback (i.e., including an even number of negative feedback interactions and/or any number of positive feedback loops) seem to be capable of causing multistationarity (Fig. 12.4b) (Angeli et al. 2004). As pointed out before, single-negative feedback reduces noise levels, and leads to homeostatic behavior (Becskei and Serrano 2000). In addition, a combination of a positive and a negative interaction is capable of inducing rhythmic oscillations. This type of regulation is a key component of both cell cycle regulation and circadian rhythms (Holtzendorff et al. 2006; Williams 2007). Finally, it was shown that a three-component negative feedback loop (repressilator) can also cause oscillatory behavior (Elowitz and Leibler 2000).

Although FBM seems to be the predominant form of bistability, one has to realize that the presence of positive feedback is no guarantee for multistationarity (Ferrell Jr 2002; Acar et al. 2005), and it is possible that systems exhibit bistability only within a certain range of parameters, such as inducer concentrations (Santillán et al. 2007, Ozbudak et al. 2004). In addition, mechanisms other than transcriptional autoregulation, such as multisite phosphorylation (Lisman 1985; Ferrell Jr 1996; Ortega et al. 2006), may be capable of inducing a bistable response.

12.5 Examples of “Natural” Multistability

12.5.1 Lactose Use in *E. coli*

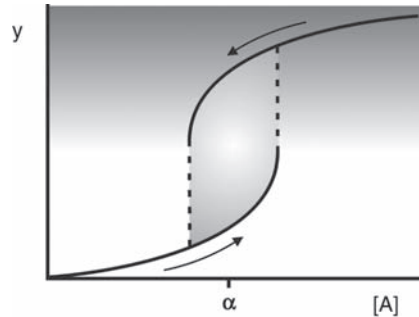
The classic example of bistability or multistability is that of the regulation of the genes responsible for lactose use in *E. coli* (Novick and Weiner 1957). In fact, regulation of the *lac* operon was the first genetic regulatory mechanism to be elucidated and is often used as the canonical example of prokaryotic gene regulation (Jacob and Monod 1961).

As a result of many years of research, the molecular mechanisms involved in the regulation of lactose use are now well known (for reviews, see Müller-Hill 1996; Laurent and Kellershohn 1999; Smits et al. 2006).

The *lac* operon comprises three genes that are required for the uptake and catabolism of lactose: *lacZ*, encoding β -galactosidase; *lacY*, encoding lactose permease; and *lacA*, encoding a transacetylase.

The expression of the *lac* operon is negatively regulated by the LacI repressor, which is inhibited by allolactose. This molecule is an isomer of lactose, converted from intracellular lactose by the constitutively expressed β -galactosidase (β -galactosidase [LacZ] enzyme in an alternative reaction to the hydrolytic reaction). LacI is also positively regulated by the cyclic-AMP receptor protein (CRP), which is activated under low sugar availability. High levels of the sugar lactose, therefore, inhibit the LacI repressor by a dual mechanism. The system demonstrates a potential positive autostimulatory loop, because the *lac* operon includes the structural gene for the permease for lactose uptake. However, β -galactosidase can metabolize both lactose and allolactose, interrupting the positive feedback loop (Fig. 12.5a),

Fig. 12.6 Schematic depiction of hysteresis. Changes in concentration of regulator are indicated by *arrows*. Depending on the starting state of the system, a cell can be in an ON (*grey*) or OFF state (*white*) at intermediate concentrations of the regulator (such as α)



transition. This phenomenon, called hysteresis (Fig. 12.6), is responsible for the observed epigenetic inheritance of the expression state over many generations (Novick and Weiner 1957; Cohn and Horibata 1959a; Cohn and Horibata 1959b). Such an unequal force essentially acts as a buffer, making the switch robust to noise, and minimizing accidental switching of the system. Under certain conditions, however, switching back from an ON state to an OFF state may still be possible. Hysteresis is not only observed for biological, but also for physical systems exhibiting bistability. In Fig. 12.6, at concentration α of inducer, the system can be either in an ON or OFF state, depending on the starting state. The state of a hysteretic system, thus, depends on its recent history. A neat analogy that describes memory by hysteresis was sketched in a review by Casadesus and D'Ari (2002). Microbiological agar is a polymer solution that at 60°C can be either liquid or solid. If the agar is melted by heating it to 100°C and then cooled down to 60°C, it will remain liquid, whereas, if solid agar at room temperature is warmed up to 60°C, it will remain solid. Thus, the state of agar at 60°C is a memory of its history. The origin of hysteresis can, for instance, lie in the stability of component within a gene-regulatory network. The hysteretic behavior of the *lac* operon, for instance, is a consequence of the abundance and stability of the LacY permease (Ozbudak et al. 2004). When little permease is present, the concentration of IPTG required to trigger the autostimulatory loop is high. In contrast, when the level of permease is high (mostly corresponding to an already induced state), cells need little IPTG to maintain high levels of *lac* expression.

Modeling of systems and networks has a long tradition in, for instance, engineering and physics, but did not become common for biological sciences until recently. This can be attributed to the relative complexity of biological systems and the inability to quantitatively analyze the data obtained from biochemical experiments. However, with the development of new experimental methods, such as single-cell analysis using fluorescent reporters, and with the increase in computational power, accurate modeling of biological systems has become possible. As a result, a number of studies combine single-cell analyses with mathematical modeling to describe the behavior of the *lac* operon at both the molecular and population level.

Modeling using (nonlinear) differential equations enabled dynamic simulations of the lactose use network and demonstrated that bistable hysteretic gene expression can be expected on the basis of reasonable biological parameters and indicated the importance of the positive feedback loop in establishing multistability (Laurent and

Kellershohn 1999; Yildirim and Mackey 2003; Yildirim et al. 2004; Ozbudak et al. 2004). Moreover, mathematically, a strong parameter sensitivity was shown. Ozbudak and colleagues demonstrated, using mathematical modeling in combination with single-cell gene expression analyses, that the lactose operon can demonstrate a graded response that can be converted to a binary response (bistability), providing a theoretical scaffold for previous unexplained observations that both states can occur in enzyme induction systems (Ozbudak et al. 2004; Biggar and Crabtree 2001). Santillan and coworkers recently showed that bistability may strongly depend on the concentrations of extracellular sugars (Santillán et al. 2007).

Whether bistability within the *lac* operon is physiologically relevant for *E. coli* remains to be tested because, when the *lac* operon is induced with lactose, bistability is not observed. Recent modeling studies, using *in silico* evolution, indicate that the wild-type *lac* operon indeed is not a bistable switch, but only shows bistability with artificial inducers (van Hoek and Hogeweg 2006).

Taken together, these studies show the quantitative power of using mathematical models to predict and identify key parameters within a (multistable) gene-regulatory network.

12.5.2 Genetic Competence in *B. subtilis*

Competence for genetic transformation, the active acquisition of extracellular DNA and the heritable incorporation of its genetic information into the host cell, is one of the stationary phase differentiation processes in which the Gram-positive bacterium *B. subtilis* can engage (for reviews, see Dubnau and Lovett 2002; Hamoen et al. 2003). Competence is the transient state governed by an extensive regulatory cascade that, ultimately, results in the activation of ComK, the master competence transcription factor. ComK is responsible for the activation of the late competence genes that constitute the DNA uptake and recombination machinery, and also stimulates its own expression (van Sinderen et al. 1995; van Sinderen and Venema 1994).

Early work has demonstrated that competent cells are physiologically distinct from their noncompetent counterparts. Nester and Stocker (1963) found that there is a lag in the increase of the number of transformants after the addition of transforming DNA to a competent culture of *B. subtilis*. In addition, the expression of a marker on the transforming DNA was not detectable during this period (Nester and Stocker 1963). They elegantly showed that, during the observed lag phase, the number of viable cells could be reduced using penicillin G, whereas the number of transformants stayed constant (Nester and Stocker 1963). These results demonstrate not only a biosynthetic latency of competent cells, but also formed the first indication that a competent culture consists of at least two subpopulations. Several later studies confirmed the presence of these subpopulations using density gradient centrifugation (Cahn and Fox 1968; Hadden and Nester 1968; Singh and Pitale 1968; Singh and Pitale 1967). These experiments demonstrated that a lower buoyant density was an inherent property of the competent cells (Cahn and Fox 1968; Hadden and Nester

1968; Dooley et al. 1971) and did not originate from or require the uptake of DNA. On the basis of density centrifugation, as well as transformation experiments using nonlinked marker genes, it was estimated that competent cells form approximately 10% of the total population (Singh and Pitale 1968; Singh and Pitale 1967; Hadden and Nester 1968; Cahn and Fox 1968). Mutagenesis of *B. subtilis* using a transposon that carries a promoterless copy of the reporter gene, *lacZ*, revealed that many genes whose deletion results in a transformation deficiency when disrupted are preferentially expressed in the band with a lower buoyant density (Albano et al. 1987; Hahn et al. 1987). In addition, the separation of competent and noncompetent cells was found to depend on the first open reading frame of the *comG* operon (Albano et al. 1987; Albano et al. 1989; Hahn et al. 1987; Albano and Dubnau 1989). To date, ComK is the only known regulator of *comG* expression (Hamoen et al. 1998; Susanna et al. 2004), and, indeed, the heterogeneity of competence could be traced back to *comK* transcription. Hahn and coworkers found that *comK-lacZ* activity was still associated with competent cells, whereas the expression of a gene acting upstream of ComK could be detected in both the light and heavy band of cells separated on a density gradient (Hahn et al. 1994). It was found that 5 to 10% of the cells in a wild-type culture express *comK-gfp* (Haijema et al. 2001), which was consistent with previous estimates (Singh and Pitale 1968; Hadden and Nester 1968; Cahn and Fox 1968). Two other important observations were made. First, the presence of the product of a late competence gene, *comEA*, was always found to coincide with *comK* expression. Second, it became clear that there are little or no intermediate levels of ComK–GFP fluorescence. This is consistent with the presence of two distinct bands, rather than a smear, in the density centrifugation experiments. When the fluorescence from a ComK-dependent *gfp* reporter in individual cells is analyzed in a quantitative manner, the resultant curve shows a bimodal distribution indicative of a bistable process (Fig. 12.1).

Recently, the origin of this bistability was experimentally addressed. The regulatory network governing competence development comprises two structural modules that could cause bistability in *comK* expression (Figs. 12.4a and 12.5b). First, ComK stimulates its own expression by directly binding as a tetramer to its own promoter region (van Sinderen and Venema 1994; Hamoen et al. 1998). As such, it forms a single positive feedback loop. Second, ComK seems to be able to form a toggle switch with a repressor of the *comK* gene, *rok* (Hoa et al. 2002). However, on the basis of two independent studies, it was found that ComK autostimulation is the critical determinant for the bistable expression pattern. Smits and coworkers (2005) demonstrated that bistability is still observed in a strain devoid of all other levels of regulation except autostimulation, and competence is still initiated in a bistable manner. In addition, it was reported that replacement of the native copy of *comK* with an inducible copy of the gene results in a graded response that depends on the level of induction (Smits et al. 2005; Maamar and Dubnau 2005). Although these experiments show that ComK autostimulation is indispensable for the bistability of competence, they do not exclude a putative ComK–Rok toggle switch (Fig. 12.5b), because the manipulations described above would also affect such a module. Maamar and Dubnau (2005) addressed this issue by introducing a *rok*

mutation in the strain with the inducible ComK. A bistable gene expression pattern was observed both the presence and absence of *rok*, but only in the presence of ComK autostimulation. Together, these data show that competence is initiated in a bistable manner, depending on ComK autostimulation.

Competence, however, is a transient differentiation process (Hadden and Nester 1968; Nester and Stocker 1963; Dubnau 1993), and it is not known how the reduction in cellular ComK levels, necessary for the escape from the competent state, is brought about. At least partially, it was found to rely on an unexplained reduction of *comK* transcription (Leisner et al 2007; Maamar et al 2007). An interesting alternative hypothesis was recently put forward, based on time-lapse microscopy (Suel et al. 2006). The authors showed that the transient differentiation observed in competence development resembles an excitable gene regulatory network and that such characteristics could be explained by a slow-acting negative feedback loop in combination with fast-acting positive autoregulation by ComK. A ComK-dependent negative feedback loop might exist (Hahn et al. 1994; Smits et al 2007), and it provides an attractive hypothesis for the transience of competence. However, investigations that are more detailed are required to address this question.

Modeling the competence regulatory pathway has provided support for the observations that intrinsic noise in *comK* expression destines cells to become competent, and also provides insight into factors that affect the probability of becoming competent and the time spent in the competent state (Maamar et al. 2007; Suel et al. 2007).

Interestingly, *B. subtilis* is not the only organism in which competence is associated with phenotypically distinct subpopulations of an isogenic culture. Competent cultures of *Streptococcus pneumoniae* are comprised of a “donor” and an “acceptor” population (Steinmoen et al. 2002; Steinmoen et al. 2003). The competent *Streptococcus* cells are thought to actively kill noncompetent cells (Guiral et al. 2005; Kreth et al. 2005). This is strikingly similar to the cannibalism described for *B. subtilis* (Gonzalez-Pastor et al. 2003), which depends on the master regulator (Spo0A) of another bistable differentiation process, sporulation (Chung et al. 1995; Veening et al. 2005). It is noteworthy that the bistable response in sporulation can be fine-tuned by regulating the phosphorylation state of the Spo0A protein (Veening et al. 2005). It can be expected that other differentiation processes affected by Spo0A potentially also demonstrate bistable behavior.

12.5.3 White–Opaque Switching in *C. albicans*

In this section, we will summarize recent advancement in the understanding of phenotypic switching in the fungus *Candida albicans*. Although the inclusion of a fungal system in a book on bacterial physiology may seem inappropriate, it is of importance to realize that phenotypic switching occurs in both fungi and bacteria (Burchard et al. 1977; Chantratita et al. 2007; Guerrero et al. 2006). However, because the molecular mechanisms underlying the switching in bacteria are relatively poorly understood, we have chosen to discuss a fungal example.

White–opaque switching, a change between two phenotypically distinct cell types, is one of the best-characterized mechanisms of generating phenotypic diversity of the opportunistic fungal pathogen *Candida albicans* (for reviews, see Bennett and Johnson 2005; Johnson 2003; Lockhart et al. 2003). The white–opaque switch was originally identified in strain WO-1, a clinical isolate of this fungus, in which switching occurs at relatively high frequency (Slutsky et al. 1987). The two phenotypic states can easily be discerned as white cells forming white, dome-shaped colonies on the plate, or opaque cells giving rise to darker and flatter colonies. In addition, white cells appear virtually spherical under the microscope, whereas opaque cells are banana shaped and demonstrate plasma membrane protrusions (Anderson and Soll 1987). It was shown that opaque cells are directly derived from white progenitor cells (Rikkerink et al. 1988). For a long time, it was assumed that *Candida* reproduces asexually, but the identification of a mating type locus similar to that of *S. cerevisiae* (Hull and Johnson 1999) led to the identification of a mating competent state (Hull et al. 2000; Magee and Magee 2000; Tsong et al. 2003). Strikingly, it was found that white–opaque switching is governed by mating-type locus homeodomain proteins (Miller and Johnson 2002). Moreover, it was shown that only opaque cells were able to mate, and that these cells were homozygous for mating type (Lockhart et al. 2002). Profiling of white and opaque cells has revealed numerous differences between the two cell types, most notably the induction of mating-type genes in opaque cells (Lan et al. 2002; Tsong et al. 2003).

Recently, two independent studies identified a single transcriptional regulator responsible for the white to opaque transition (Zordan et al. 2006; Huang et al. 2006). This regulator, WOR-1, is preferentially expressed in opaque cells and, therefore, repressed in *a/α* cells (Lan et al. 2002; Tsong et al. 2003). It was reported that, on induction of an ectopic copy of the gene encoding the regulator, white cells are converted to opaque cells, suggesting that the WOR-1 bypasses the repression by the *a/α* repressor in heterozygous cells (Huang et al. 2006; Zordan et al. 2006). WOR-1 is capable of stimulating its own expression by binding directly to its own promoter (Zordan et al. 2006), which results in a bistable expression pattern (Huang et al. 2006). Two important observations were made by Zordan et al. (2006). First, they found that, in the presence of WOR-1 autostimulation, white cells were stably converted into opaque cells by a pulse in expression from an ectopic copy of the regulator gene. Second, they observed that, in the absence of an autostimulatory loop, continuous expression of WOR-1 was required to maintain the opaque phenotype.

In conclusion, white–opaque switching is repressed by the *a/α* repressor in heterozygous cells (Lockhart et al. 2002; Soll et al. 2003). In cells homozygous for mating type (which may still be white), a basal level of expression from the *WOR-1* locus will cause some cells to switch to the opaque state when the threshold for WOR-1 autostimulation is reached (Fig. 12.5c). It is of importance to realize that the bistable switching only refers to WOR-1 expression states, and mating type DNA rearrangement and homozygosity/heterozygosity merely set the conditions under which bistability can occur.

Feedback-based bistability may be a common mechanism for switches such as the white–opaque switch. *Myxococcus xanthus* displays a switch between a tan and a

yellow phenotype, for instance, and, although this is sometimes referred to as phase variation, no genomic inversion could be demonstrated and the molecular mechanisms remain elusive (Burchard et al. 1977; Laue and Gill 1994). Similarly, the molecular mechanisms behind phenotypic switching of *Cryptococcus neoformans* (Guerrero et al. 2006) and *Burkholderia pseudomallei* (Chantratita et al. 2007) remain to be established. Interestingly, developing cells of this organism display a bimodal distribution in the expression of the *dev* locus (Russo-Marie et al. 1993), which has been suggested to originate from a positive feedback loop (Viswanathan et al 2007).

12.6 Perspectives and Implications of Bistability

Phenotypic variation, in general, and bistability, in particular, are widespread phenomena in the bacterial realm. This has implications for medicine, food industry, biotechnology, and bioinformatic analyses.

In medicine and the food industry, the existence of subpopulations of cells resistant to conventional treatments has a large impact on the control of bacterial infections and contaminations. Latent bacterial infections, the occurrence of persisters, and tolerance of pathogens to a multitude of drugs are increasingly problematic (Lewis 2007).

Persistence is the well-known phenomenon that, after treatment of a bacterial strain with a specific antibiotic, rapid killing of the vast majority of cells is observed, followed by a more complex and slow killing of the remaining cells. Eventually, a small proportion of the cells can survive. This behavior has been described for *Staphylococcus aureus* treated with penicillin in 1944 (Bigger 1944). Persistence is a bet-hedging strategy because, under optimal conditions, a majority of cells proliferate quickly and a small subpopulation suppresses growth. During times of stress, e.g., the presence of antibiotics, these persistent cells can prevent extinction of the entire population (Kussell et al. 2005). Persisters exhibit reduced translation and topoisomerase activity, and/or reduced cell wall biosynthesis, as a result of which, the targets of many antibiotics are blocked. Thus, these cells cannot be easily killed, at the cost of nonproliferation (reviewed in Lewis 2007). The molecular mechanism underlying persistence remained obscure until recently. The use of optical microscopy for single-cell analysis showed that persistence in *E. coli* is a phenotypic switch, and that at least two types of persistence can be identified: stationary phase-induced persistence (type I) and spontaneous persistence (type II) (Balaban et al. 2004). The generation of persisters is most probably caused by stochastic fluctuation and exceeding thresholds, just as described in Sect. 12.4 of this review for the mechanisms underlying multistationarity (Lewis 2007). Possibly, the occurrence of persisters can be reduced by factors that enhance the switching rate from persister to normal growth, providing possible solutions for drug use that is more effective (Balaban et al. 2004).

Phenotypic tolerance (resistance caused by a multifactorial phenotypic adaptation) is closely related to persistence. When bacteria are exposed to bactericidal

concentrations of antimicrobial compounds, their sensitivity gradually decreases, and certain subpopulations survive. Usually there is also cross-tolerance to other antimicrobials (Wiuuff et al. 2005). Acquired nisin resistance in Gram-positive bacteria was shown to be fully reversible when cells were allowed to grow again in media without the bacteriocin, indicating that the increased resistance was not genetically determined (Kramer et al. 2006). Interestingly, phenotypic heterogeneity was found to occur when cells are subjected to subinhibitory (or sublethal) amounts of bacteriocin. *Listeria monocytogenes* challenged by nisin or leucocin 4010 developed two subpopulations, as observed by fluorescence ratio imaging microscopy (Hornbaek et al. 2006). One of the subpopulations showed cells with a dissipated pH gradient (Δ pH), whereas the other subpopulation maintained the Δ pH. The study shows that it is of great importance to use the appropriate dose of the antimicrobial compound, taking into account its bioavailability, when it is applied for food preservation.

In addition to these phenomena, it has been noted that *Salmonella* growing in host macrophages demonstrates fast- and slow-dividing subpopulations (Abshire and Neidhardt 1993). It remains to be established whether this is related to the production of antimicrobial compounds by the macrophages.

Although the mechanisms responsible for the observed phenotypic heterogeneity are not yet elucidated, it is tempting to speculate that epigenetic regulatory mechanisms such as bistability may play a role. It is interesting to note that, after selection of epigenetically determined phenotypes, the survivor cells retain the ability to generate the same phenotypic variability (Fig. 12.7). Indeed, it was shown through mathematical modeling that cells with a variable phenotype have increased fitness under fluctuating environmental conditions (Thattai and van Oudenaarden 2004).

Besides the occurrence of resistant microbial contaminants in production systems, bistability can also affect processes such as protein production. As extensively discussed in Sect. 12.5.1, the all-or-none enzyme induction of the lactose use operon has become a paradigm for bistability because it can occur for enzyme induction, although similar behavior has been also described for lactose use in *Salmonella enterica* serovar Typhimurium (Tolker-Nielsen et al. 1998). Arabinose-inducible systems are widely used for protein overproduction and purification, where the existence of a nonproducing subpopulation is unwanted. However, arabinose use in *E. coli* also demonstrates a heterogeneous population distribution, most likely as a result of bursts in the synthesis of the arabinose permease, because of stochastic changes in the conformation of a transcriptional activator (Morgan-Kiss et al. 2002). To increase the yields of protein production, derivatives of production strains, in which the production of permease is uncoupled from enzyme induction and the positive feedback loop is, thus, effectively removed, have been constructed (Khlebnikov et al. 2002; Khlebnikov et al. 2001; Khlebnikov et al. 2000). In addition, strains devoid of a dedicated arabinose uptake and degradation system were constructed, in which a mutated LacY permease transports arabinose into the cells (Morgan-Kiss et al. 2002).

From a fundamental point of view, the existence of subpopulations has implications for the interpretation of data from bulk assays, as indicated in Sect. 12.1. It

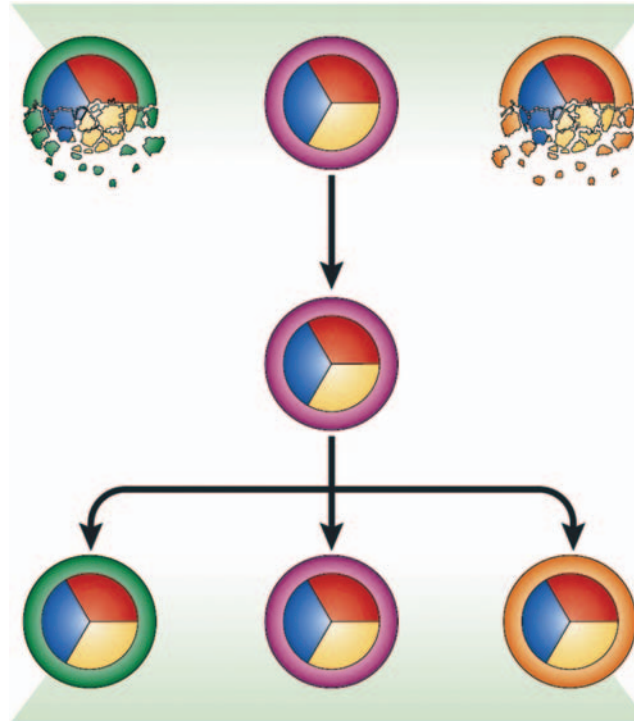


Fig. 12.7 Survival of heterogeneous phenotypes. Three genetically identical cells display phenotypic variability. The genetic content of the cells is indicated in the inner circle with primary colors. The phenotype is depicted by the colors of the outer rings. In a selective condition, only cells with a particular phenotype will survive. However, because the genetic content has not been altered, the survivor cell has the ability of regenerating the same phenotypic variability (Reproduced from Smits et al. 2006 with permission of the publisher)

would be of great interest to see whether it will become possible to discriminate the behavior of small subpopulations from the large majority of cells with the help of bioinformatics, and, thus, improve the interpretation of high-throughput data, such as DNA microarrays or proteome studies. Additionally, the study of the output from artificial gene networks, modeling studies, and classic molecular and cell biological methods will aid in the reconstruction and connectivity of gene regulatory networks *in silico*.

Highly Recommended Readings

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